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Single Probe for Real-time Simultaneous Monitoring of Neurochemistry and Direct-current Electrocorticography

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1 **ABSTRACT**

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3 We report a novel single neural probe for real-time simultaneous monitoring of multiple
4 neurochemicals and direct-current electrocorticography (DC-ECOG). A major advance of
5 this probe is the inclusion of two iridium oxide reference electrodes to improve sensor
6 accuracy. The ECOG reference electrode is identical to the ECOG recording electrodes to
7 significantly improve DC stability, while the reference for electrochemical sensors has
8 10-fold lower polarization rate to minimize the small current-induced drift in the
9 reference electrode potential. *In vitro*, the single probe selectively measured oxygen
10 ($r^2=0.985\pm0.01$, concentration range=0-60 mmHg, limit of detection= 0.4 ± 0.07 mmHg)
11 and glucose ($r^2=0.989\pm0.009$, concentration range=0-4 mM, limit of detection= 31 ± 8 μ M)
12 in a linear fashion. The performance of the single probe was assessed in an *in vivo* needle
13 prick model to mimic sequelae of traumatic brain injury. It successfully monitored the
14 theoretically expected transient brain oxygen, glucose, and DC potential changes during
15 the passage of spreading depolarization (SD) waves. We envision that the developed
16 probe can be used to decipher the cause-effect relationships between multiple variables of
17 brain pathophysiology with the high temporal and spatial resolutions that it provides.

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19 **Keywords:** neurochemical; direct-current electrocorticography; spreading depolarization;
20 iridium oxide reference electrode; real-time monitoring
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1. Introduction

The brain is the most complex organ in the human body made up of a network of billions of neurons. The communications between the neurons are inherently electrical and chemical in nature through approximately 100 billion neurons with 100 trillion synapses. Hence, there is a considerable need in neuroscience research for *in situ*, simultaneous acquisition of the electrical and chemical signals. After acute injury to the brain, neurons at the damage epicenter depolarize, and this depression of activity spreads outward in waves through the cortex (Ayata and Lauritzen, 2015; Dreier, 2011; Leao, 1944). Decades of research show that cortical spreading depolarization (SD) can cause progressive lesion growth after ischemic, hemorrhagic, or traumatic brain injury by increasing tissue metabolic burden and diminishing perfusion (Chase, A., 2014; Hartings et al., 2009; Hartings et al., 2011; Hinzman et al., 2014; Lauritzen et al., 2011; Takano et al., 2007). Detection of such processes through neuromonitoring offers the prospect of targeting patient therapies to detected intracranial pathology. However, not all pathologic brain activity is harmful, and accurate multimodal monitoring of SD and other events is needed to determine which ones require treatment. Furthermore, simultaneous electrical and chemical measures in the same brain tissue location can provide insight to cause-effect relationships among the multiple variables of interest and inform the best therapeutic approach.

Previous attempts at simultaneous electrocorticography (ECoG) and neurochemical signal recordings were based on the use of multiple, separate electrodes, which limits spatial and temporal resolution since separate sensors are not located within the same microenvironment (Dash et al., 2009; Naylor et al., 2011; Odijk et al., 2015; Rogers et al., 2013). Consequently, the development of a single probe capable of simultaneously detecting electrical and chemical signals is of great significance. Implantable micromachined microelectrode arrays represent a versatile and powerful tool to achieve this *in vivo* (Fekete, 2015; Tseng and Monbouquette, 2012; Wise et al., 2008). The techniques allow for the development of smaller electrodes that provide improved spatial and temporal resolution as well as multiple sensing electrodes with precisely defined geometries. Micro-electrochemical sensors allow the continuous, precise and fast *in situ* measurement of metabolic parameters directly within the tissue (Cordeiro, C.A., 2015; Hinzman et al., 2015; Kealy et al., 2013; Rocchitta et al., 2013; Wei et al., 2014; Weltin et al., 2014).

Recently, several neural probes have been developed for simultaneous electrophysiological and neurochemical signal recordings (Johnson et al., 2008; Morales-Villagrán et al., 2008; Song et al., 2012; Wei et al., 2015). By placing both ECoG recording electrodes and electrochemical sensors in the area of interest, near real-time monitoring of multiple parameters is possible. However, it is challenging to measure multiple variables simultaneously, especially for DC-ECoG (**direct-current electrocorticography**), since crosstalk between sensors introduces noise and instability. For example, DC-ECoG recording electrodes should have a very low polarization rate and stable baseline for detecting the DC shift of SD. Currently, microelectrodes for SD recording are limited to glass micropipettes with Ag/AgCl wire due to their non-polarizable property (Hinzman et al., 2015; Odijk et al., 2015; Rogers et al., 2013). No study to date has examined the accuracy and stability of micromachined probes for

1 simultaneous DC-ECOG and electrochemical signal recordings.

2 The reference electrode is a key component for both ECOG electrodes and
3 electrochemical sensors (Shinwari et al., 2010). However, these electrodes are not usually
4 integrated into a single sensing probe. In previous work, an Ag/AgCl wire, screw, or
5 platinum wire was used as a remote reference for the ECOG electrode and was placed in a
6 comparable but inactive area such as the contralateral hemisphere, musculature, or
7 subdermal space (Lewitus et al., 2011; Muthuswamy et al., 2005). Most electrochemical
8 sensors used for *in vivo* studies also rely on a separate reference electrode, usually a
9 Ag/AgCl wire (Thomas et al., 2009; Wassum et al., 2012). Ag/AgCl, however, has been
10 shown to cause widespread necrosis in biocompatibility studies (Stensaas and Stensaas,
11 1978) and the implantation of multiple electrodes can cause more damage to the brain. A
12 single probe combining all sensing and biocompatible reference electrodes can overcome
13 this important issue, **which is particularly important in the clinical setting**. Such an
14 integrated probe for *in vivo* application not only reduces tissue damage, but may also
15 reduce noise arising from external sources and within the brain, providing improved
16 signal-to-noise ratios (SNR) and detection limits (Li et al., 2009a; Peteu et al., 1996;
17 Tolosa et al., 2013).

18 Previous studies have shown that iridium oxide (IrOx) electrodes can be a suitable
19 replacement for Ag/AgCl wire reference electrodes for electrochemical sensors and can
20 be used as a quasi-reference electrode for *in vivo* neuroscience applications (Tolosa et al.,
21 2013; Yang et al., 2004;). IrOx exhibits excellent biocompatibility and mechanical
22 stability; it is stable over a range of potentials and in the presence of the ions and other
23 components of brain extracellular fluid. Although the IrOx potential shows strong pH
24 dependence (Ges et al., 2005), the small dynamic range of normal brain pH (7.15-7.4)
25 makes this issue negligible (less than 30 mV potential drift) in most cases. However, IrOx
26 has drawbacks as well. Compared to Ag/AgCl, IrOx microelectrodes have much higher
27 impedance which not only slows the response of the potentiostats, but also increases the
28 susceptibility of the system to environmental noise. In addition, unlike the nearly ideal
29 non-polarization liquid-solid interface which Ag/AgCl forms in solutions, IrOx is a
30 polarizable material, such that the passage of small currents can alter its potential. So far,
31 the stability of the IrOx electrode potential was only studied in PBS (**Phosphate-buffered**
32 **saline**) solution; its stability as a reference electrode under continuous electrochemical
33 sensor operation has not been reported.

34 In this paper, we present a new micromachined single probe for real-time simultaneous
35 monitoring of multiple neurometabolic and DC-ECOG signals. ECOG microelectrodes are
36 designed to be placed in gray matter to detect cortical SD, whereas electrochemical
37 sensors are designed to be placed in white matter to measure the focal brain oxygen and
38 glucose concentrations simultaneously. In place of separate Ag/AgCl reference
39 electrodes, two biocompatible IrOx reference electrodes are incorporated on-probe to
40 achieve improved DC stability and accuracy. The ECOG reference electrode is identical
41 to the ECOG recording electrodes so that both electrodes detect the same background
42 noise and hence increase the DC stability and SNR. The reference electrode for the
43 electrochemical sensors has **10-fold lower polarization rate** than the ECOG reference to
44 minimize the open circuit potential drift due to small current-induced polarization. To
45 assess the performance of the developed single probe, it was tested *in vivo* using a needle
46 prick model that induces SD, thereby mimicking the electrophysiologic and

neurochemical changes of developing brain injury. We demonstrate that this single probe can accurately monitor brain glucose, oxygen tension, and DC-ECoG simultaneously.

2. Materials and methods

2.1 Reagents and apparatus

All chemicals were analytical reagent grade or higher purity and were used as received. Chitosan (MW~10⁵, 75-85% deacetylation), glucose oxidase (GOD) from *Aspergillus niger* (EC 1.1.3.4), glutaraldehyde (25% in water), β -D-glucose, potassium chloride (K₂PtCl₆), sulfuric acid (99.999%), *o*-phenylenediamine (*o*PD), iridium tetrachloride hydrate, oxalic acid dehydrate (99%), hydrogen peroxide (30% solution in water), anhydrous potassium carbonate, and phosphate buffered saline (pH 7.4) were purchased from Sigma-Aldrich. All solutions were prepared with distilled water collected from Millipore Direct-Q.

All electrochemical preparations necessary for electroplating platinum nanoparticles, iridium oxide, and calibration of oxygen and glucose biosensors were performed using a potentiostat (PalmSens, Palm Instruments BV, the Netherlands). The model 215 benchtop research-grade pH/mV meter (Denver Instrument Corp., USA) was used to measure open circuit potential (OCP) of iridium oxide relative to the Ag/AgCl electrode (MF-2052; Bioanalytical Systems Inc., USA). The oxygen tensions at the difference calibration solution were measured using a precision temperature-compensated fiber-optic oxygen measurement system (OXYMICRO-Fiber-optic sensor; World Precision Instruments).

The analog outputs from both the ECoG and electrochemical sensors are sampled at 1 KHz and recorded and analyzed with a Powerlab 16/SP analog/digital converter and CHART-7 software (ADInstruments, USA). AC-ECoG signals are generated by applying band-pass filtering at 0.5-50 Hz on the original DC-coupled signals.

2.2 Probe design and fabrication

Standard microfabrication processes were used for development of the single neural probe. Briefly, the polyimide PI2611 (HD Microsystems) was spin coated at 3000 rpm for 30 seconds on a 4 inch silicon wafer. The polyimide was cured under nitrogen atmosphere at 450 °C for 60 minutes resulting in a 6 μ m thick layer. After exposure to oxygen plasma (100 W, 30 mTorr) for 30 seconds, the metal layer (Ti/Au 120Å/1200Å) was deposited using e-beam metal evaporator (Temescal FC1800, BOC Edwards Temescal, USA) and patterned by photolithography to build the microelectrodes, interconnection lines and connection pads. A second PI2611 layer was then spin-coated to form a 6 μ m thick layer to insulate the interconnection lines. The cured second polyimide layer was etched in oxygen plasma (200 W, 30 mTorr) for 10 minutes. This etching step opened the microelectrodes and connection pads. Platinum nanoparticles were electrochemically deposited on all of the opened microelectrodes by cyclic voltammetry in 0.5 M H₂SO₄ solution containing 2 mM K₂PtCl₆ with cycling between 0.4 V and - 0.25 V at 50 mV/s (Li et al., 2009a). Iridium oxide was further electrochemically deposited onto the Pt modified microelectrodes (all reference and

1 ECoG recording electrodes) by cycling between 0 V and 0.6 V at 50 mV/s in the plating
2 solution described by Marzouk *et al* (Marzouk et al., 1998). 0.2-0.5 μ l of the biocatalytic
3 mixture consisting of GOD-cerium oxide-glutaraldehyde-chitosan (GOD: 25U/ μ l; cerium
4 oxide: 1mg/ml; chitosan: 1%; glutaraldehyde: 1.25%) in a ratio of (1:1:5:50) was coated
5 on the working electrode of the glucose biosensor and cured for 12 hours at 4 °C. After
6 that, the working electrodes for the glucose and oxygen sensors were electro-coated in
7 phosphate buffer pH 7.4 containing 300 mM oPD solution by applying a potential of 0.7
8 V for 10 minutes. The flexible film was cut to size (width=1.5 mm) and spirally rolled to
9 form a single neural probe (inner diameter=0.65 mm; outer diameter=0.7 mm) based on
10 our previous work (Li et al., 2009b; Li et al., 2007). The in-plane structure of the
11 developed single probe is shown in Figure 1A and the assembled probe is shown in
12 Figure 1B.

13
14 **Figure 1**

15
16 *2.3 Headstage construction and measurement*

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18 We developed a headstage and sensor interface circuit board for the 2-channel
19 electrochemical biosensors and the 2-channel ECoG microelectrodes. These provide the
20 signal conditioning functions for the sensors while minimizing the sensor cable length
21 that can otherwise degrade the signal-to-noise ratio (SNR). Figure 1C shows the circuit
22 diagram of the headstage (21 mm $\times$ 40 mm). The ECoG recording circuit (**A₁₋₂: AD8220,**
23 **Analog Devices Inc.; R₁₋₃: 1 M Ω and C₁₋₃: 1 nF**) comprises RC filters for removing
24 ambient noise and the instrumentation amplifiers for signal amplification with a gain of
25 25. In order to achieve a high quality DC-ECoG recording operation, i.e. high SNR, the
26 amplifier has been carefully selected to achieve low noise, high common-mode rejection
27 ratio (CMRR) and high input impedance. The amplifier has typical total noise referred to
28 input of 5 μ V_{pp} from 0.1-10 Hz and minimum CMRR of 86 dB at DC for gain = 1 and
29 input impedance of 10 T Ω || 5 pF. The electrochemical sensing is achieved by using a
30 potentiostat circuit (**A₃₋₆: OPA2140, Texas Instrument Inc.; R₄₋₅: 1 k Ω ; C₄: 100 pF; C₅₋₆:**
31 **10 nF**). The circuit is capable of operating two electrochemical biosensors at the same
32 time. The electrochemical sensing circuit has to perform **sensing** at current level in nA
33 range. **To minimize the nA-level current sensing error**, a **high-precision** Op Amp with
34 typical offset voltage of 30 μ V and very low input bias current of $\pm$ 0.5 pA is used for
35 realizing the circuit. The current readout from the working electrodes is achieved by
36 using a switch-based transimpedance amplifier. Analog switches with leakage current <1
37 pA (**MAX327: Maxim Integrated, Inc.**) are used for this purpose.

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40 *2.4 In vivo measurements*

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42 Six adult male Sprague-Dawley rats weighing 300 – 450 gram were used in the
43 experiments. The rats were housed in a temperature-controlled room on a 12-h light/dark
44 cycle, with food and water available *ad libitum* in accordance with the standards of the
45 Association of Assessment and Accreditation of Laboratory Animal Care International.
46 This study was approved by the Institutional Animal Care and Use Committee (IACUC)

of The Feinstein Institute for Medical Research. All procedures were performed during the light cycle and carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources).

The rats were anesthetized (5% isoflurane for induction and 2.5-3% isoflurane for maintenance during the surgery) and placed in a stereotaxic frame with a base heating pad, and supplied with a steady flow of medical air. A craniotomy was made over parietal cortex of the right hemisphere ($\sim 0.8 \text{ mm}^2$; ML: +2 $\rightarrow$ +3 mm; AP: -2 $\rightarrow$ -3 mm; DV: -4.5 mm). The dura was incised with a 30 gauge needle and the exposed brain was kept moist with physiologic saline irrigation. The probe was implanted into the cortex avoiding major vasculature. A small burr-hole was drilled in the same hemisphere 3 mm anterior to the probe for needle pricks. A Ag/AgCl wire (diameter=0.25 mm) were fixated to the left hemisphere skull 6 mm from the probe as alternative reference electrode for the electrochemical biosensors. The probe, screw and wire were cemented into place using dental cement. A waiting period of 60 minutes followed the neural probe implantation. During recordings, rats were mounted in a stereotaxic frame and anesthesia was maintained at 1.5-2.0 % isoflurane and body temperature was maintained at 37 °C with a closed-loop temperature controller. At the end of experiments, rats were deeply anesthetized and euthanized by decapitation.

3. Results and discussion

3.1 Potential crosstalk and solution

All microelectrodes on the neural probe are in direct contact with the conductive media. There is a chance that the low-frequency current generated from the electrochemical biosensors affects the ECoG recording and the electrochemical reference electrodes if these sensors are in close proximity. Figure 2A illustrates the crosstalk paths between the electrochemical biosensors and the ECoG electrodes via their equivalent circuit model. I_N represents neuronal activity current, and I_L stands for the leakage current crosstalk from the electrochemical biosensors and environment. The ECoG recording and its reference electrode are modeled as a capacitor in parallel with a resistor. R_F is faradaic resistor and C_D the double-layer capacitor. The subscript M refers to the recording electrode and R the reference electrode. R_E is the equivalent resistor of the electrolyte. The crosstalk pathways are highlighted by the dashed line. Normally, only the current from neuronal activity, I_{N1} and I_{N2} , are present. The leakage current, I_L , becomes appreciable when both sensors are physically close. Under this condition, I_L diverts from the electrochemical sensors to the ECoG recording, ECoG reference and electrochemical sensor reference electrodes. It also causes current-induced polarization in these electrodes. If the ECoG recording and reference electrode are identical (material and impedance), the common-mode interference from current-induced polarization can be cancelled. On the other hand, if the leakage current-induced polarizations to the ECoG recording and reference electrode are different due to mismatches in electrode characteristics, the interference can cause slowly-varying drift to appear on ECoG recording outputs. If too large, the drift confounds identification of shifts caused by neural activity, such as SD. For the

1 electrochemical sensor, errors can be produced if the reference electrode potential drifts
2 due to current-induced polarization.

3 If all electrodes are made from non-polarizable materials such as Ag/AgCl, the errors
4 induced by the current crosstalk are negligible. However, IrOx is a polarizable material
5 and hence vulnerable to crosstalk. To mitigate this, separate reference electrodes are used
6 for the electrochemical sensors and the ECoG recording electrodes. This approach
7 permits the reference electrodes for the electrochemical sensor and the ECoG electrode to
8 have different electrical properties. The electrochemical sensor reference electrode was
9 designed to have low impedance, minimizing voltage drift. On the other hand, the ECoG
10 reference electrode was designed to match the ECoG recording electrodes, thereby
11 achieving superior DC stability and noise performance.

12 13 Figure 2

14 15 16 3.2 Current-induced polarization

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18 Current-induced polarization occurs when current flows to the polarizable
19 microelectrodes and causes potential drift. Therefore, low polarization rates are desirable
20 for DC-ECoG and reference electrodes. Current-induced polarization tests were
21 performed on electrodes (microelectrodes: 0.045 mm^2 ; large IrOx electrode: 0.45 mm^2)
22 with different materials to assess polarization effects. Constant current pulses of $\pm 5 \text{ nA}$
23 were applied to the Pt, IrOx, Ag/AgCl microelectrodes and large IrOx electrodes and
24 their voltage responses were recorded. The constant current reverses its polarity every
25 100 seconds. Eight voltage responses for each microelectrode were recorded and
26 averaged. Figure 2B shows the measurement results. Ag/AgCl wire, a common choice for
27 reference electrodes in biosensors due to its very small voltage response to the current,
28 was used as a test reference electrode. A small DC-voltage of $0.015 \pm 0.007 \text{ mV}$ was
29 observed at 100 s for Ag/AgCl microelectrodes. By comparison, Pt microelectrodes
30 showed a large DC-voltage at $0.418 \pm 0.046 \text{ mV}$, indicating that Pt is not a suitable
31 material for DC-biopotential recording and reference electrode. IrOx showed small DC-
32 voltage of $0.064 \pm 0.028 \text{ mV}$ and $0.006 \pm 0.002 \text{ mV}$ for small and large IrOx
33 microelectrodes, respectively, which are close to DC-voltage of Ag/AgCl
34 microelectrodes. Thus, IrOx is a desirable material for DC-biopotential recording and
35 reference electrodes. In our single neural probe, the small IrOx microelectrodes are used
36 as ECoG recording and reference electrodes, since use of the same material provides for
37 superior noise immunity and reduces potential interference from electrochemical sensors.
38 The large IrOx electrode is used as the reference electrode for electrochemical sensors.

39 40 3.3 AC noise and DC stability

41
42 The AC noise and DC stability of the ECoG microelectrodes were assessed when on-
43 probe IrOx microelectrode was used as an ECoG reference electrode in comparison with
44 a conventional separate Ag/AgCl reference electrode. ECoG signal was measured while
45 electrochemical oxygen and glucose sensors were operated simultaneously. The recorded
46 DC-ECoG signal was filtered offline to obtain the AC component (0.5 to 50 Hz), as

1 shown in Figure 3A. The AC noise level of the ECoG signal measured with the separate
2 Ag/AgCl reference electrode (i) and with on-probe IrOx reference electrode (ii) are ~40
3 μV_{pp} and ~25 μV_{pp} , respectively. The matched design (identical material and impedance)
4 of the ECoG recording and on-probe ECoG reference electrodes thus showed reduced
5 noise performance, although differences of 10- μV s may be negligible with baseline
6 signals of ± 0.5 mV.

7 Figure 3B shows the recorded DC-ECoG signal during SD. SD is associated with
8 massive depolarization of neurons and redistribution of ions across cell membranes. The
9 DC-ECoG signal with the separate reference electrode (i) has large DC drift in
10 comparison to the DC-ECoG signal with on-probe reference electrode (ii). This is
11 because only the ECoG recording electrode has experienced the crosstalk from the
12 electrochemical sensors when its reference electrode is placed remotely. The crosstalk
13 can cause polarization at the ECoG recording electrode and show itself as a DC drift at
14 the outputs. On the other hand, the ECoG signal with on-probe reference electrode
15 experiences more than 5-fold lower DC drift. The identical material and closer location of
16 the on-probe reference electrode and the ECoG recording electrode allow the crosstalk to
17 polarize at similar levels, which cancel in the ECoG recording circuit.

18 The DC shifts of SD waves may be cancelled or superimposed with distortion if the
19 distance between the ECoG recording and reference electrodes is very close. The distance
20 effect was investigated by considering the ECoG recording electrode 2 in Figure 1A as an
21 ECoG recording electrode and another four electrodes at different distances as ECoG
22 reference electrodes (*in plane* center to center distance: 0.6 mm, 1.8 mm, 3.3 mm, 6.5
23 mm). Working electrodes for glucose and oxygen sensors were electroplated with IrOx
24 for this test. If the detected AC baseline signals using separate Ag/AgCl reference
25 electrode was considered 100%, the measured signals are decreased to approximately
26 15%, 40%, 70% and 97% when ECoG recording electrode 1, oxygen sensor working
27 electrode, glucose sensor working electrode and ECoG reference electrode were used as
28 reference electrodes, respectively. No noticeable SD waveform distortions were recorded
29 in current probe design.

31 Figure 3

34 3.4 Analytical characterization and calibration of the biosensors

35
36 The performance of electrochemical sensors is critically dependent on the reference
37 electrode and its ability to maintain a stable potential. Previous studies have shown that
38 IrOx can be a suitable replacement for Ag/AgCl wire reference electrodes and could be
39 used for quasi-reference electrodes in the brain. To investigate the stability of the
40 potential of IrOx as a reference electrode under continuous electrochemical sensor
41 operation (3-electrode biosensor), its open circuit potential (OCP) was measured in 1 mM
42 glucose solution (pH 7.4). The OCP response of the developed IrOx electrodes is shown
43 in Figure 4A. Twelve IrOx electrodes were tested for each case and all the potentials
44 were recorded after 1 day stabilization in PBS (pH 7.4). When the small IrOx electrode
45 (surface area=0.045 mm²) was not connected to the potentiostat, its potential drift was
46 less than 0.8 ± 0.3 mV (n=12) over 12 hours, matching previously reported data. However,

the drift increased by more than 27-fold (21.9 ± 3.7 mV; $n=12$) when the small IrOx electrode was used as a reference electrode for the glucose biosensor with current outputs of ~ 12 nA. The drift was decreased to 2.5-fold (8.7 ± 2.1 mV; $n=12$) for the large IrOx electrode (surface area = 0.45 mm²). These results demonstrate that IrOx can be used as a quasi-reference electrode for electrochemical sensors, but the impedance should be lowered to minimize the polarization-induced potential drift for long-term continuous monitoring. This can be achieved by either increasing the electrode surface area or thickness.

In vitro calibrations of oxygen and glucose sensors were conducted using constant potential amperometry. The bias potentials of -0.75 V and 0.45 V were applied to the oxygen and glucose sensors *versus* the IrOx reference electrode (corresponding to ~ -0.6 and 0.6 V *versus* Ag/AgCl), respectively. The current outputs of sensors were averaged over a 5 minute period at each baseline level and the limit of detection (LOD) of sensors was calculated as 3 times the standard deviation of the baseline noise over a 1 minute period. *In vitro* calibration of oxygen sensors was carried out in PBS equilibrated at 37 °C with specified gas mixtures containing 1-8% oxygen. Figure 4B shows the calibration curve for oxygen sensors. Good linearity with a correlation coefficient of 0.985 ± 0.01 and sensitivity of 0.9902 ± 0.13 nA/mmHg ($n=17$) in the linear range from 0 to 60 mmHg were observed. Figure 4C shows the calibration curve for the glucose sensors at 37 °C with oxygen tension of 38 mmHg. They have sensitivity of 11.864 ± 0.21 nA/mM in the linear range from 0 to 4 mM with a linear coefficient of 0.989 ± 0.009 ($n=11$). The limit of detection for oxygen and glucose sensors is 0.4 ± 0.07 mmHg and 31 ± 8 μ M, respectively.

Figure 4

3.5 Multimodal monitoring of spreading depolarization

The simultaneous operation of all sensors on the single probe was tested *in vivo* by mechanically inducing a single SD wave with a needle prick to the rat cerebral cortex. Two needle pricks were performed with a 1 hour interval in four rats. Representative recordings are shown in Figure 5. Successful induction of the SD wave was confirmed by a negative shift of the DC-ECOG potential (2.1 ± 0.3 mV; $n=8$) and a transient suppression of the spontaneous AC-ECOG activity, the characteristic signatures of mass tissue depolarization. During the SD, the brain oxygen tension showed a biphasic response with an initial increase (27.4 ± 4.2 mmHg; $n=8$) followed by persistent decrease (10.2 ± 3.2 mmHg; $n=8$) below baseline values (15.8 ± 9.6 mmHg; $n=8$). The brain glucose concentration showed a transient decrease from baseline (41 ± 22 μ M; $n=8$) during the SD and almost recovered to its baseline value (0.61 ± 0.17 mM; $n=8$). These experimental data are in general agreement with the changes in local oxygen tension and glucose concentration that have been reported previously during SD in the normal brain (Hashemi et al., 2008; Parkin et al., 2005; Piilgaard and Lauritzen, 2009). However, measurement of all three variables simultaneously has not previously been possible. Thus, the findings presented here highlight the potential of the single neural probe for the study of cerebral

function and metabolism through accurate monitoring of multiple variables in the same region of brain tissue.

Figure 5

4. Conclusion

In the present study, a single neural probe for real-time simultaneous monitoring of multiple neurometabolic and DC-ECoG signals has been successfully developed. The use of two on-probe iridium oxide reference electrodes greatly improved the DC stability of baseline ECoG signals and also increased the accuracy of electrochemical sensors by minimizing the current-induced drift, thereby improving the capacity for long-term monitoring. As proof-of-concept, the performance of the probe was tested *in vivo* using a needle prick to induce SD and associated changes in tissue oxygen and glucose. **Successful simultaneous recording of these variables highlights the potential of this technology for use in characterizing other important neurochemical correlates of brain activity in both normal and pathologic states.**

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Figure Captions:

Figure 1. Microphotograph of (A) the *in-plane* structure of the single neural probe and (B) fully assembled probe for rat brain monitoring. ECoG recording microelectrodes are placed in gray matter to detect spreading depolarization, while biosensors are placed in white matter to measure multiple cerebral metabolic parameters. (C) Interface circuit diagram of the headstage for 2-channel electrochemical sensors and 2-channel ECoG microelectrodes. Inset in panel (C) shows the developed headstage prototype. Inclusion of the reference electrodes of biosensors and ECoG microelectrodes on the same probe improves the overall signal accuracy. The developed probe is capable of simultaneously monitoring multiple neurochemicals and DC-ECoG in real time. WE: working electrode; RE: reference electrode; CE: counter electrode

Figure 2. (A) Electrical model to explain the potential crosstalk between the electrochemical microsensors and ECoG electrodes and (B) current-induced polarization test for Pt, Ag/AgCl and IrOx microelectrodes. The constant current pulses of ± 5 nA were applied to the Pt, IrOx, Ag/AgCl microelectrodes and large IrOx electrodes and their voltage responses were recorded. The constant current reverses its polarity every 100 seconds. Eight voltage responses for each microelectrode were recorded and averaged. RE: reference electrode

Figure 3. Comparison between the separate Ag/AgCl electrode (i) and the on-probe IrOx reference electrode (ii): (A) AC noise and (B) DC stability. ECoG electrodes with on-probe reference electrode showed better AC noise and DC stability.

Figure 4. Analytical characterization of the biosensors: (A) open circuit potential change of IrOx reference electrodes (small IrOx: surface area of 0.045 mm^2 ; large IrOx: surface area of 0.45 mm^2 ; $n=12$). Small IrOx reference electrodes experienced at least 27-fold and 2.5-fold higher potential drift if they were used as reference electrodes for glucose sensors. Calibration curves of (B) Oxygen sensor and (C) glucose sensor using on-probe IrOx reference electrode (large IrOx).

Figure 5. Multimodal monitoring of spreading depolarization in an *in vivo* needle prick model: transient brain oxygen tension and glucose concentration changes during single episode of cortical spreading depolarization are shown. (A) DC-ECoG (bandpass 0-50 Hz), (B) AC-ECoG (bandpass 0.5-50 Hz), (C) brain oxygen tension and (D) brain glucose concentration. Data in A, B, C, and D are chosen from one rat and selected from 8 needle pricks.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5